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The endonuclease Ankle1 requires its LEM and GIY-YIG motifs for DNA cleavage in vivo

Andreas Brachner[1], Juliane Braun[1], Medini Ghodgaonkar[2], Dennis Castor[2], Livija Zlopasa[1], Veronika Ehrlich[3], Josef Jiricny[2], Josef Gotzmann[1], Siegfried Knasmüller[3] and Roland Foisner[1,*]

[1]Max F. Perutz Laboratories, Medical University of Vienna, Dr. Bohr-Gasse 9, A-1030, Vienna, Austria
[2]Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057, Zurich, Switzerland
[3]Institute of Cancer Research, Inner Medicine I, Medical University of Vienna, Borschkegasse 8a, A-1090, Vienna, Austria
*Author for correspondence (roland.foisner@meduniwien.ac.at)

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Summary

The LEM domain (for lamina-associated polypeptide, emerin, MAN1 domain) defines a group of nuclear proteins that bind chromatin through interaction of the LEM motif with the conserved DNA crosslinking protein, barrier-to-autointegration factor (BAF). Here, we describe a LEM protein annotated in databases as 'Ankyrin repeat and LEM domain-containing protein 1' (Ankle1). We show that Ankle1 is conserved in metazoans and contains a unique C-terminal GIY-YIG motif that confers endonuclease activity in vitro and in vivo. In mammals, Ankle1 is predominantly expressed in hematopoietic tissues. Although most characterized LEM proteins are components of the inner nuclear membrane, ectopic Ankle1 shuttles between cytoplasm and nucleus. Ankle1 enriched in the nucleoplasm induces DNA cleavage and DNA damage response. This activity requires both the catalytic C-terminal GIY-YIG domain and the LEM motif, which binds chromatin via BAF. Hence, Ankle1 is an unusual LEM protein with a GIY-YIG-type endonuclease activity in higher eukaryotes.

Key words: Chromatin, DNA damage, GIY-YIG endonucleases, LEM domain, Nuclear envelope

Introduction

Polypeptides containing a LEM (for lamina-associated polypeptide, emerin, MAN1) domain (Lin et al., 2000) comprise a protein family with important and essential functions in nuclear architecture, mitosis, cell signaling and gene expression (Gruenbaum et al., 2005; Schirmer and Foisner, 2007; Wagner and Krohne, 2007; Anderson and Hetzer, 2008). The LEM domain is a structural motif of 45 amino acids that folds as two α-helices (Laguri et al., 2001) and binds to barrier-to-autointegration factor (BAF) (Furukawa, 1999; Cai et al., 2001; Shumaker et al., 2001), an essential DNA crosslinking protein in metazoans (Umland et al., 2000; Zheng et al., 2000; Margalit et al., 2007).

The LEM protein family in mammals includes the well-characterized proteins lamina-associated polypeptide 2 (LAP2), emerin and MAN1 (Schirmer and Foisner, 2007; Wagner and Krohne, 2007), the MAN1-related protein LEM domain-containing protein 2 (LEM2) (Brachner et al., 2005; Chen et al., 2006; Ulbert et al., 2006; Huber et al., 2009) and a partially characterized testis-specific protein, LEM domain-containing protein 1 (LEMD1, also known as LEM5) (Lee and Wilson, 2004; Yuki et al., 2004). Two of these proteins (emerin and LEM2) are conserved in *Caenorhabditis elegans* (Lee et al., 2000; Gruenbaum et al., 2002; Liu et al., 2003). Most of the characterized LEM proteins are transmembrane proteins of the inner nuclear membrane where they interact with the nuclear lamina (Schirmer and Foisner, 2007; Wagner and Krohne, 2007). LEM protein–BAF complexes are involved in post-mitotic nuclear assembly and in chromatin organization in *C. elegans* (Liu et al., 2003; Margalit et al., 2005) and in mammalian cells (Haraguchi et al., 2001; Dechat et al., 2004; Shimi et al., 2004). In addition, several LEM domain proteins bind to and regulate transcription factors and signaling molecules such as β-catenin (Markiewicz et al., 2006), Smads (Lin et al., 2005; Pan et al., 2005; Jiang et al., 2008), germ-cell-less (gcl) (Nili et al., 2001; Holaska et al., 2003) and retinoblastoma protein (Markiewicz et al., 2002; Dorner et al., 2006). Mutations in genes encoding emerin, MAN1 and LAP2α have been linked to a number of human pathologies (Vlcek and Foisner, 2007; Wagner and Krohne, 2007; Worman and Bonne, 2007; Chi et al., 2009), reflecting their multiple and diverse functions.

In silico analyses of mammalian genomes have identified previously unknown genes encoding proteins predicted to be members of the LEM family, originally termed LEM3 and LEM4 (Lee et al., 2000; Lee and Wilson, 2004) and annotated in databases as Ankyrin repeat and LEM domain-containing proteins 1 and 2, respectively. In this study, we report the first biochemical and cell biological characterization of Ankle1 [also known as LEM3, Ankyrin repeat domain-containing protein 41 (ANKRD41) and FLJ39369], an evolutionary conserved non-membrane-bound LEM protein that shuttles between nucleus and cytoplasm and is predominantly expressed in hematopoietic tissues and cells.

Intriguingly, the Ankle1 C-terminus contains an enzymatically active GIY-YIG endonuclease domain, previously described as the characteristic feature of a subgroup of the homing endonuclease superfamily. Homing endonucleases are encoded within 'selfish' group I introns or inteins. They catalyze their lateral transfer and integration into intronless homologous alleles

in the host genome (Stoddard, 2005; Edgell, 2009; Stoddard, 2011). A large number of these enzymes have been identified in all three biological kingdoms: archae, bacteria and eukaryotes (Belfort and Roberts, 1997; Sokolowska et al., 2011). The GIY-YIG homing endonucleases that have been characterized in detail include I-TevI in T4 phage (Bell-Pedersen et al., 1991; Van Roey et al., 2002) and I-SceI in the yeast mitochondrial genome (Perrin et al., 1993; Moure et al., 2003). The GIY-YIG motif has also been found in enzymes other than homing endonucleases, such as UvrC, a protein involved in nucleotide excision repair in bacteria (Yoakum and Grossman, 1981; Truglio et al., 2005). Only a few genes in higher eukaryotes are known to encode proteins that contain a GIY-YIG motif: the recently characterized gene encoding human endonuclease SLX1, which is involved in structure-specific DNA repair (Fekairi et al., 2009; Svendsen et al., 2009), the transpositionally active *Penelope*-like elements in *Drosophila virilis* (Pyatkov et al., 2004; Evgen'ev and Arkhipova, 2005; Schostak et al., 2008) and the as-yet-uncharacterized human gene encoding Ankle1 (Dunin-Horkawicz et al., 2006).

Results

Ankle1 is highly conserved in metazoans

ANKLE1 is also termed *ANKRD41*, *FLJ39369* or *LEM3* and was annotated in databases on the basis of different computational screens for genes encoding proteins that contain either a LEM domain, Ankyrin repeats or a GIY-YIG motif (Lee et al., 2000; Lee and Wilson, 2004; Dunin-Horkawicz et al., 2006). Assembly and alignment of Ankle1 sequences from numerous species obtained from the ENSEMBL and NCBI databases (supplementary material Fig. S1A) revealed a conserved domain organization of the protein (see Fig. 1): two to four (depending on the species) N-terminal Ankyrin repeats, a central putative LEM domain, and a predicted C-terminal GIY-YIG motif within a highly conserved C-terminal sequence stretch termed 'PB014249' in the Pfam database (www.pfam.sanger.ac.uk). This latter C-terminal domain including the GIY-YIG motif showed the highest homologies, ranging from ~70% conserved residues among vertebrates to ~40% conservation between nematodes and mammals (Fig. 1; supplementary material Fig. S1B). Unlike the domain architecture, the predicted molecular weights of Ankle1 vary considerably, ranging from 58 kDa in mouse to 102 kDa in zebrafish (Fig. 1). Human Ankle1 has 615 residues and a predicted molecular weight of 65 kDa. The variability in size is particularly evident in the region between the Ankyrin repeats and the LEM domain, indicating that the distance between these domains might be less important for its activity. By contrast, the spacing between the LEM domain and the GIY-YIG motif was preserved and might thus be crucial for the function(s) of Ankle1.

Human Ankle1 is expressed in a tissue-restricted manner

We analyzed the expression levels of Ankle1 in human tissues and cell lines by semiquantitative RT-PCR using primers for the 3′ region of *Ankle1* cDNA. Analysis of mRNA samples from a collection of adult human tissues revealed predominant *Ankle1* mRNA expression in bone marrow. Expression was also high in fetal liver, fetal spleen and fetal thymus, the primary organs of hematopoiesis during intrauterine development (Fig. 2A), suggesting that Ankle1 might be involved in hematopoiesis-specific processes. In addition, we observed expression in a panel of lymphoma- and leukemia-derived tumor cell lines (ARH77, CCRF, DAUDI, K562, RAJI, RAMOS and REH), whereas sarcoma- and carcinoma-derived lines (HACAT, HeLa, LSWW, MCF-7, SW-480, T-98-G and U2OS) expressed low or undetectable levels of *Ankle1* mRNA (Fig. 2A). RT-PCR analysis of human bone marrow mRNA samples using a further upstream forward primer (Fig. 2B) identified a minor, slightly smaller isoform of Ankle1, originating from alternative splicing within exon 5 (see also Q8NAG6-1 in the Uniprot database). Interestingly, the splice variant, which we termed Ankle1b, lacks half (amino acids 375–400) of the putative LEM domain (Fig. 2B).

Next, we tested Ankle1 protein expression, using rabbit polyclonal antibodies raised against the N-terminal recombinant mouse Ankle1 fragment (amino acids 95–260, antiserum number 136). Although the antibodies readily detected ectopically expressed Ankle1 in western blots of HeLa cell lysates (Fig. 2C) (human Ankle1–V5, mouse Ankle1–V5) as well as in immunofluorescence microscopy (see later), endogenous Ankle1 was not detectable in various human or mouse lymphoma cell lines that contain abundant *Ankle1* mRNA levels (Fig. 2C). We

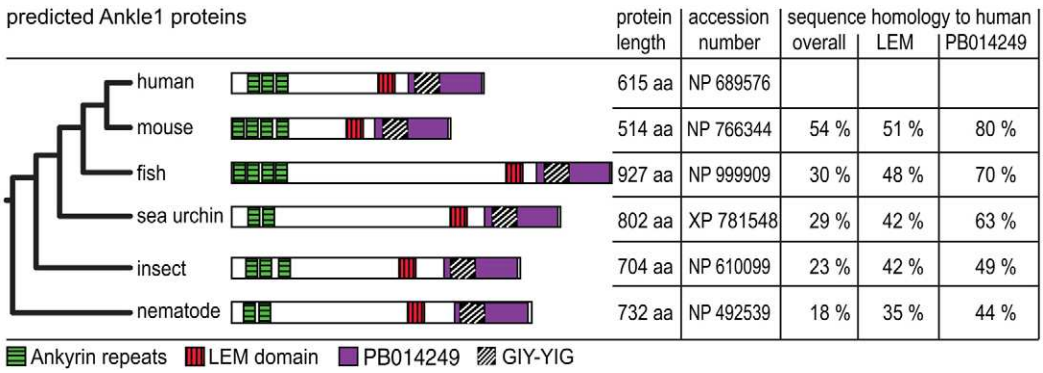

Fig. 1. Ankle1 is a LEM protein conserved among metazoans. Comparison of the predicted domain organization of Ankle1 orthologs from various species representing major metazoan clades: *Homo sapiens* and *Mus musculus* (mammals), *Danio rerio* (vertebrates), *Strongylocentrotus purpuratus* (echinodermata), *Drosophila melanogaster* (arthropods) and *Caenorhabditis elegans* (nematodes). Ankyrin repeats are shown as green boxes, the LEM motif as red boxes and the C-terminal PB014249 sequence as violet boxes. The predicted GIY-YIG motif is marked as a black-hatched box. Number of amino acids (aa) of full-length proteins, database accession numbers and the sequence homologies of various domains to human Ankle1 are shown on the right.

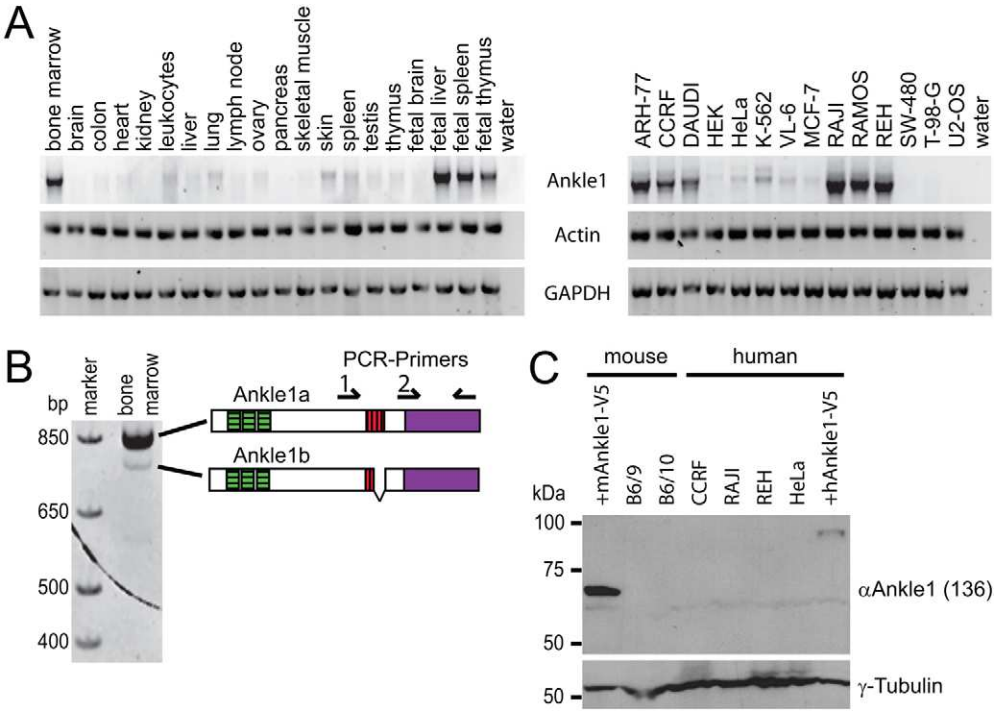

Fig. 2. Ankle1 is predominantly expressed in human hematopoietic tissues and cell lines. (**A**) Representative agarose gels showing semiquantitative RT-PCR products amplified from human tissue and cell line samples. Actin and GAPDH were used as controls for equal loading. (**B**) Resolution of human Ankle1 PCR products from human bone marrow on polyacrylamide gels revealed a low-abundance smaller Ankle1 isoform (Ankle1b). Position of PCR primers are indicated (primer 1 was used for Ankle1 splice variant identification; primer 2 for expression analyses). The identity of the isoform was verified by sequencing. (**C**) Representative western blot detecting Ankle1 protein in total lysates of HeLa cells expressing ectopic human or mouse Ankle1–V5. Tubulin was probed as a loading control.

obtained similar results with antibodies raised against a highly conserved peptide within the C-terminal region (amino acids 388–402) of mouse Ankle1 (data not shown). Thus, the antisera clearly detected ectopic Ankle1 protein in mammalian cells in both the unfolded and native state (western blotting and immunofluorescence microscopy, respectively), but we could not detect endogenous Ankle1. Therefore, we assumed that endogenous Ankle1 protein levels are strictly controlled and expressed at levels below the detection limit of our assays.

Ankle1 binds to BAF

A characteristic feature of LEM proteins is their interaction with BAF via the LEM motif. In order to test whether Ankle1 also binds BAF, we performed in vitro pull-down assays using recombinant 6×histidine-tagged BAF (BAF–His). Bacterial lysates containing BAF–His were mixed with *Escherichia coli* cell lysates expressing recombinant full-length Ankle1, an Ankle1 fragment (Ankle1ΔCT) lacking the C-terminus including the predicted LEM domain and, as a positive control, the N-terminal part of the LEM protein emerin (EmerinΔTM), which was shown to bind BAF (Lee et al., 2001). BAF–His was precipitated using magnetic Ni particles and the supernatant and pellet fractions were analyzed by western blotting. BAF–His efficiently co-precipitated full-length Ankle1 and emerin, whereas Ankle1ΔCT remained in the unbound fraction (Fig. 3A), indicating that the C-terminal half of Ankle1 binds BAF.

To test whether the LEM domain mediates Ankle1–BAF interaction, we performed pull-down assays with bacterially expressed full length 6×His-V5-tagged Ankle1 (Ankle1–His-V5) and the corresponding Ankle1 splice variant lacking a part of its LEM domain (Ankle1b–His-V5). Precipitation of Ankle1–His-V5 with Ni beads brought down a significant fraction of

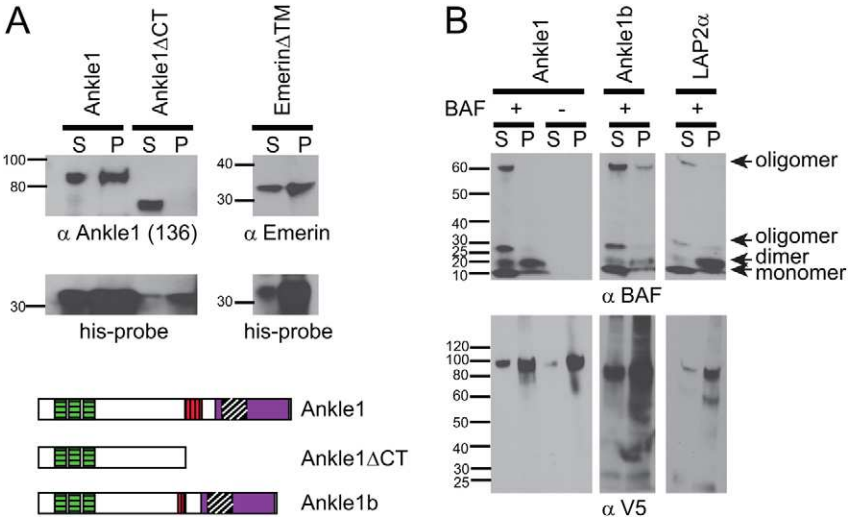

Fig. 3. Co-precipitates of BAF and Ankle1 in vitro. (**A**) BAF-6×His was mixed with *E.coli* lysates containing recombinant Ankle1, Ankle1ΔCT or an N-terminal emerin fragment EmerinΔTM and precipitated using Ni beads. BAF-bound proteins were detected by western blotting using antiserum '136' (Ankle1), India His-Probe (BAF) and monoclonal antibody MANEM5 to emerin. Molecular masses are indicated in kiloDaltons. Ankle1 constructs used in the assays are shown at the bottom; for key see Fig. 1. (**B**) Recombinant 6×His-V5-tagged Ankle1, Ankle1b or LAP2α were incubated with bacterial lysates containing untagged human BAF. His-tagged prey proteins were precipitated using Ni beads and bound BAF was detected by western blotting using antibodies to the V5 tag and BAF. Note that BAF forms various homomeric complexes even under denaturating conditions (arrows indicating monomeric, dimeric and oligomeric states). S, supernatant fraction (10% of input); P, precipitated fraction (50% of precipitated proteins).

recombinant, untagged BAF from bacterial cell lysates (Fig. 3B). Predominantly dimeric BAF was detected in the Ankle1 pellet fraction, whereas monomeric and oligomeric BAF mainly remained in the supernatant fraction. Similar results were obtained in the positive control using the LEM protein LAP2α, which efficiently pelleted BAF dimers. By contrast, Ankle1b–His-V5, lacking a functional LEM domain, precipitated only trace amounts of BAF, and very little dimeric BAF was detected in the supernatant and pellet fractions. Overall, these findings show that Ankle1 interacts directly with BAF via its LEM domain, and indicate that binding of BAF to the LEM domain may favor BAF dimerization.

Ankle1 shuttles between cytoplasm and nucleus

Because antisera failed to detect endogenous Ankle1 protein (Fig. 2C), we expressed GFP-tagged human Ankle1 in a lymphoma and a carcinoma-derived cell line, expressing or lacking endogenous *Ankle1* mRNA, respectively (Fig. 1A). In both lymphoma-derived RAMOS cells and HeLa cells we observed predominant cytoplasmic localization of GFP–Ankle1 by fluorescence microscopy (Fig. 4A). Identical results were obtained using a C-terminal GFP tag (data not shown) or a small tag such as V5 (Fig. 4B). Because in silico analyses of the Ankle1 primary sequence predicted potential nuclear export and nuclear localization signals (NES and NLS, Fig. 4A, scheme), we hypothesized that Ankle1 shuttles between the nucleus and cytoplasm. To test this hypothesis, we blocked Crm-dependent nuclear export using the drug Leptomycin B (Kudo et al., 1998). After 1 hour of Leptomycin B treatment, most of the tagged Ankle1 was accumulated in the nucleus in both HeLa and RAMOS cells (Fig. 4). Thus, Ankle1 is actively transported in and out of the nucleus and shows a predominant cytoplasmic localization at steady state.

Ankle1 has nuclease activity that elicits DNA damage signaling in the nucleus

Given that Ankle1 contains a putative GIY-YIG-type endonuclease domain, we performed a series of in vitro and in vivo assays to test for endonuclease activity. Recombinant Ankle1 purified from HEK cells cleaved supercoiled plasmid DNA into relaxed circular (nicked) and linearized DNA molecules (Fig. 5A), suggesting that Ankle1 possesses in vitro nuclease activity. Several conserved residues in the GIY-YIG motif (Y453, G488, E551 in human Ankle1, see supplementary material Fig. S3A) were previously identified as crucial for the formation of the catalytic surface of the nuclease (Van Roey et al., 2002; Truglio et al., 2005; Lagerback and Carlson, 2008). Point mutations of either of these residues abrogated the in vitro DNA cleavage activity of Ankle1 (Fig. 5A). Thus, we concluded that Ankle1 possesses intrinsic endonuclease activity mediated by its C-terminal canonical GIY-YIG motif.

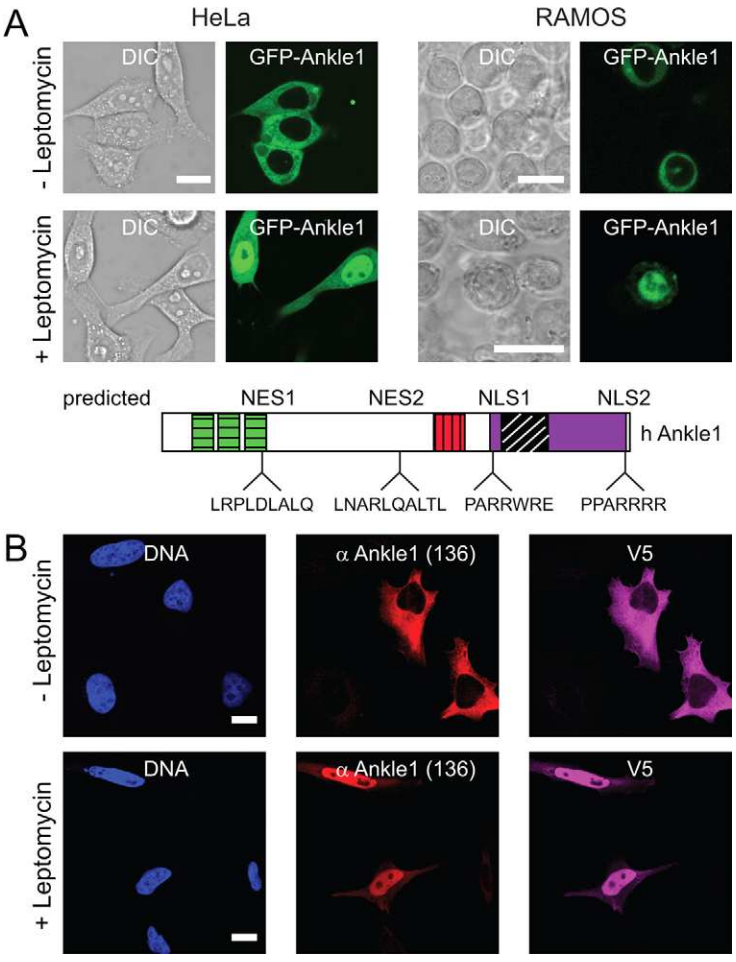

Fig. 4. Human Ankle1 shuttles between nucleus and cytoplasm. (**A**) GFP-tagged Ankle1 was expressed in HeLa or RAMOS cells and imaged by live-cell microscopy (GFP) and differential interference contrast (DIC) microscopy before (−) and after (+) treatment with Leptomycin for 1 hour. Nuclear export signals (NES) and nuclear localization signals (NLS) identified by in silico prediction software are indicated. (**B**) HeLa cells were transfected with human Ankle1–V5, and processed for immunofluorescence microscopy before and after 3 hours of Leptomycin treatment. Scale bars: 10 μm.

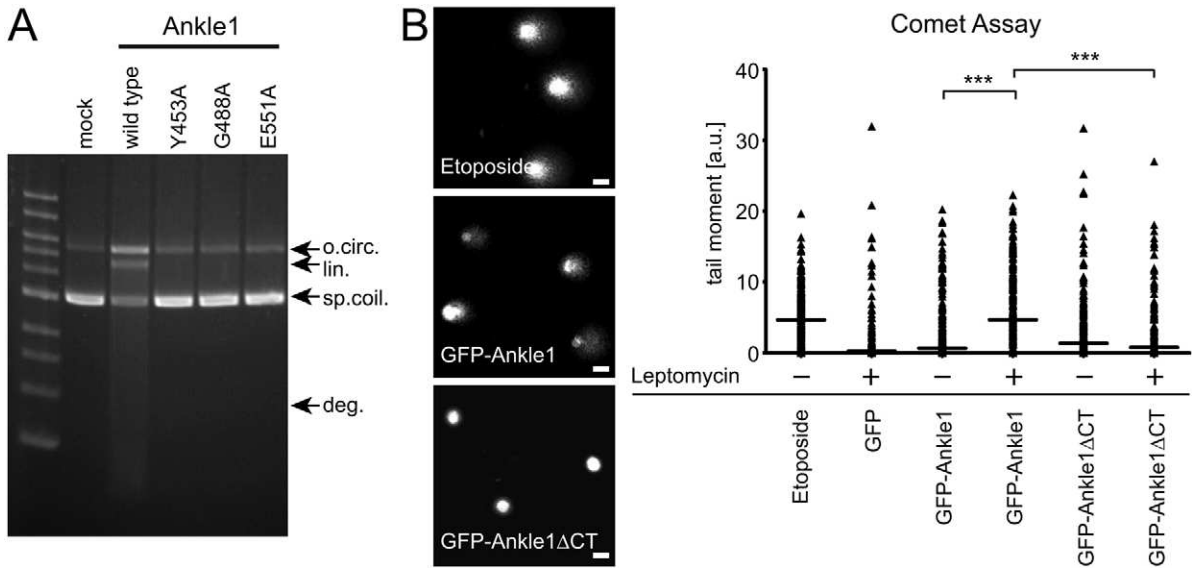

Fig. 5. Ankle1 cleaves DNA in vitro and in vivo. (**A**) Purified wild-type and mutated HA-Strep-Ankle1 expressed in HEK cells was incubated with supercoiled pFastBac1 plasmid and the DNA species separated by agarose gel electrophoresis. Arrows indicate different intermediate steps of plasmid degradation (o.circ, open circle or nicked; lin, linear; sp.coil, supercoiled; deg, degraded). (**B**) Representative images of ethidium-bromide-stained nuclei in a Comet assay. Scale bars: 10 μm. Comets were measured using the Comet Assay IV software, quantified and visualized in Graphpad Prism. Median values are indicated as horizontal lines. ***$P<0.001$ indicates statistically significant differences in median values between populations. Comet formation (i.e. DNA fragmentation) was evaluated in single cells of three independent cell populations ($n>50$ each).

In order to test whether Ankle1 also cleaves DNA in cells in vivo, we performed COMET (i.e. single-cell gel electrophoresis) assays, an established method for detecting genomic DNA fragmentation in vivo at the single-cell level. Expression of GFP–Ankle1 in HeLa cells for 24 hours caused little DNA damage, similar to the negative control cells expressing GFP only (Fig. 5B). By contrast, treatment of cells with Leptomycin, causing GFP–Ankle1 accumulation in the nucleus, induced DNA cleavage to a similar extent as cells treated with the topoisomerase II inhibitor Etoposide, a drug known to induce massive DNA double-strand breaks (Sullivan et al., 1986). Leptomycin treatment of cells expressing GFP or a truncated Ankle1 lacking the C-terminal GIY-YIG motif did not induce DNA breaks (Fig. 5B).

In order to find out whether Ankle1-mediated DNA cleavage activates DNA damage signaling, we analyzed the expression and localization of components of the DNA damage response pathway in GFP–Ankle1-expressing cells before and after Leptomycin treatment. In more than 90% of cells ($n>100$) nuclear accumulation of GFP–Ankle1 following Leptomycin treatment caused relocalization of 53BP1 to intranuclear foci (Schultz et al., 2000), phosphorylation of Histone 2A.X (γH2A.X) and activation of the downstream effector kinase Chk2 (pChk2) (Fig. 6A), which was similar to the results in Etoposide-treated cells (Fig. 6B). Identical results were obtained in cells expressing Ankle1–V5 (supplementary material Fig. S2). Leptomycin treatment of GFP-expressing cells did not induce these markers (Fig. 6C), indicating that accumulation of Ankle1 in the nucleus, but not Leptomycin treatment and/or transfection, activated the DNA damage signaling pathway. Nuclear accumulation of a C-terminally truncated Ankle1 protein missing the GIY-YIG motif (GFP–Ankle1ΔCT) did not activate the DNA damage response pathway (Fig. 6D). These observations indicate that accumulation of Ankle1 in the nucleus causes DNA cleavage, thereby activating the DNA damage response pathway.

The LEM domain of Ankle1 is required for DNA cleavage in vivo

Defined DNA binding and targeting motifs described in homing endonucleases, such as zinc-finger domains, helix-turn-helix motifs and DNA minor groove-binding α-helical structures (Derbyshire et al., 1997; Kowalski et al., 1999; Liu et al., 2006; Carter et al., 2007), are not known in Ankle1 therefore we hypothesized that the LEM motif of Ankle1 might be involved in correct targeting of the protein. In order to test whether the LEM domain is involved in Ankle1-mediated DNA cleavage, we measured γH2A.X staining intensity in mixed cultures of cells expressing either full-length Ankle1 or the LEM-domain-deficient variant Ankle1b. To correlate the extent of DNA damage (γH2A.X staining intensity) with Ankle1 expression levels in individual cells, we also measured the intensity of the V5 staining. Unlike Ankle1b, full-length Ankle1 was expressed from a vector also expressing a GFP marker, allowing us to discriminate between cells expressing full-length Ankle1 or Ankle1b in the mixed culture (Fig. 7). Immunofluorescence analyses of mixed cell populations were performed before and after Leptomycin treatment (Fig. 7). The fluorescence intensity of the V5-specific signal in the nucleus of Leptomycin-treated cells was measured in GFP-positive cells (i.e. cells expressing full-length Ankle1–V5) and GFP-negative cells (expressing Ankle1b–V5) and plotted over the intensity of γH2A.X-specific fluorescence (Fig. 7). DNA damage (measured via γH2A.X intensity) increased with increasing levels of full-length Ankle1, whereas only low levels of γH2A.X were detectable in Ankle1b-expressing cells independently of the expression level (Fig. 7).

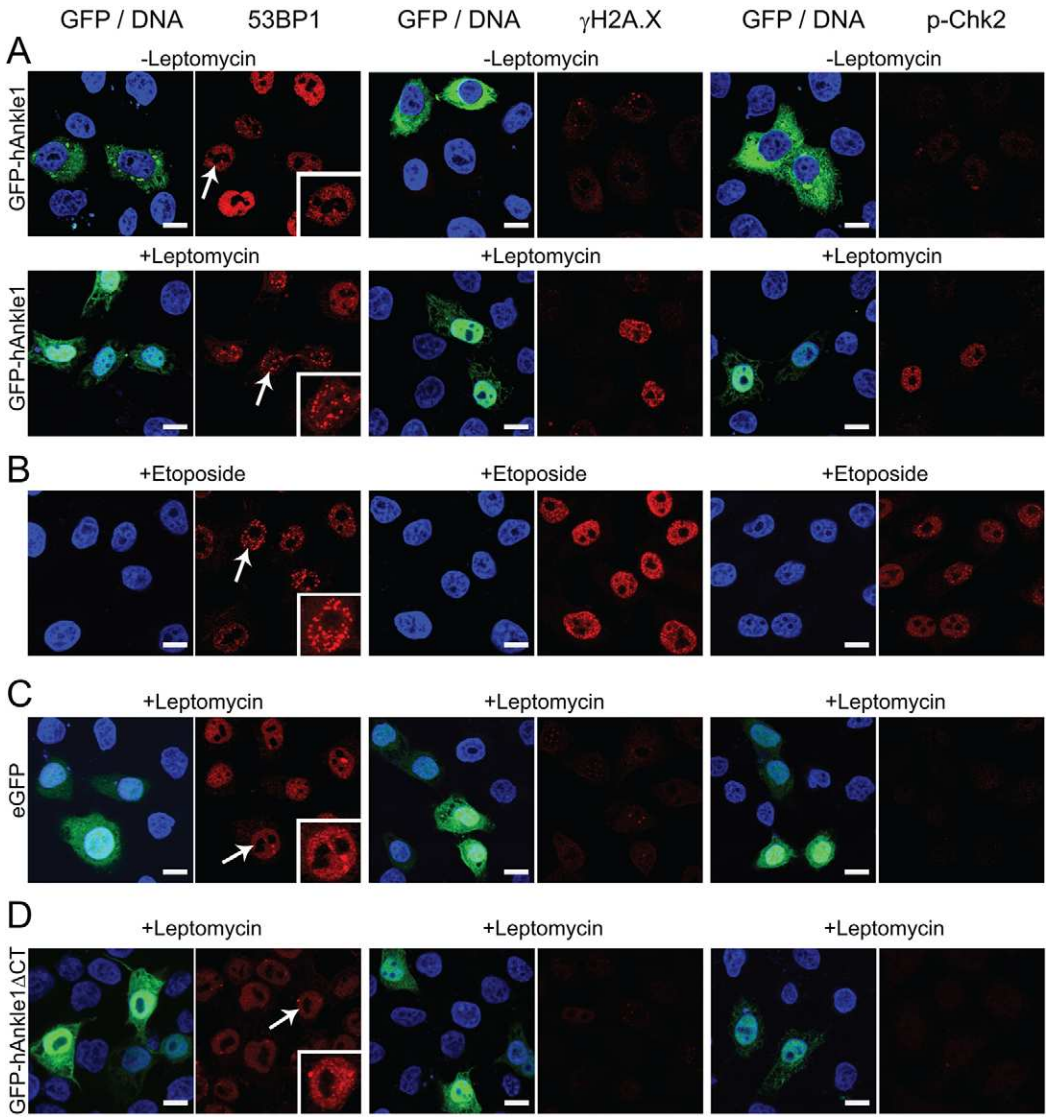

Fig. 6. Accumulation of ectopic Ankle1 in the nucleus causes DNA damage. (**A**–**D**) HeLa cells were transiently transfected with GFP–Ankle1 (A), GFP (C) or GFP–Ankle1ΔCT (D) and treated with Leptomycin as indicated, or incubated with Etoposide (B). All samples were processed in parallel for confocal immunofluorescence microscopy. Fixed cells were stained for 53BP1, γH2A.X or phosphorylated Chk2 using specific antibodies (all red), DNA was stained with DAPI (blue), GFP and GFP-fusion proteins are shown in green. Arrows indicate the cells shown in the insets. Representative images out of at least three independent experiments. Scale bars: 10 μm.

Thus, the presence of a functional LEM motif is required for the Ankle1-mediated activation of the DNA response pathway, suggesting that this domain might be involved in targeting Ankle1 to chromatin in vivo.

Discussion

In this study we performed biochemical and cell biological analyses of a previously uncharacterized human LEM protein, annotated as Ankle1 in databases. The LEM domain of Ankle1 interacts with BAF, confirming that Ankle1 is a bona fide LEM protein. Apart from its interaction with BAF, Ankle1 also showed four unexpected novel and unique properties among the characterized LEM protein family members: (1) Ankle1 expression is largely restricted to hematopoietic tissues, whereas most other LEM proteins in mammals are widely expressed (Theodor et al., 1997; Ellis et al., 1998; Lin et al., 2000; Brachner et al., 2005). Only *LEMD1* (also known as *LEM5*) (Lee and Wilson, 2004) has so far been reported to be expressed in a tissue-restricted manner (testis) (Yuki et al., 2004). (2) Although all analyzed LEM proteins contain a LEM motif close to their nucleoplasmic N-terminus (Wagner and Krohne, 2007), the LEM domain of Ankle1 is located in the middle of the polypeptide. (3) Ankle1 lacks transmembrane domains and shuttles in and out of the nucleus. Non-membrane-bound localization among characterized LEM proteins has only been reported for two isoforms of the LAP2-encoding gene, *LAP2α* (Dechat et al., 1998; Dechat et al., 2004) and *LAP2ξ* (Shaklai et al., 2008). All other studied LEM proteins contain one or two transmembrane domains and localize to the inner nuclear membrane. (4) The most intriguing and exceptional feature of Ankle1, however, is an enzymatically active GIY-YIG motif that has been previously described in homing endonucleases and a few other proteins,

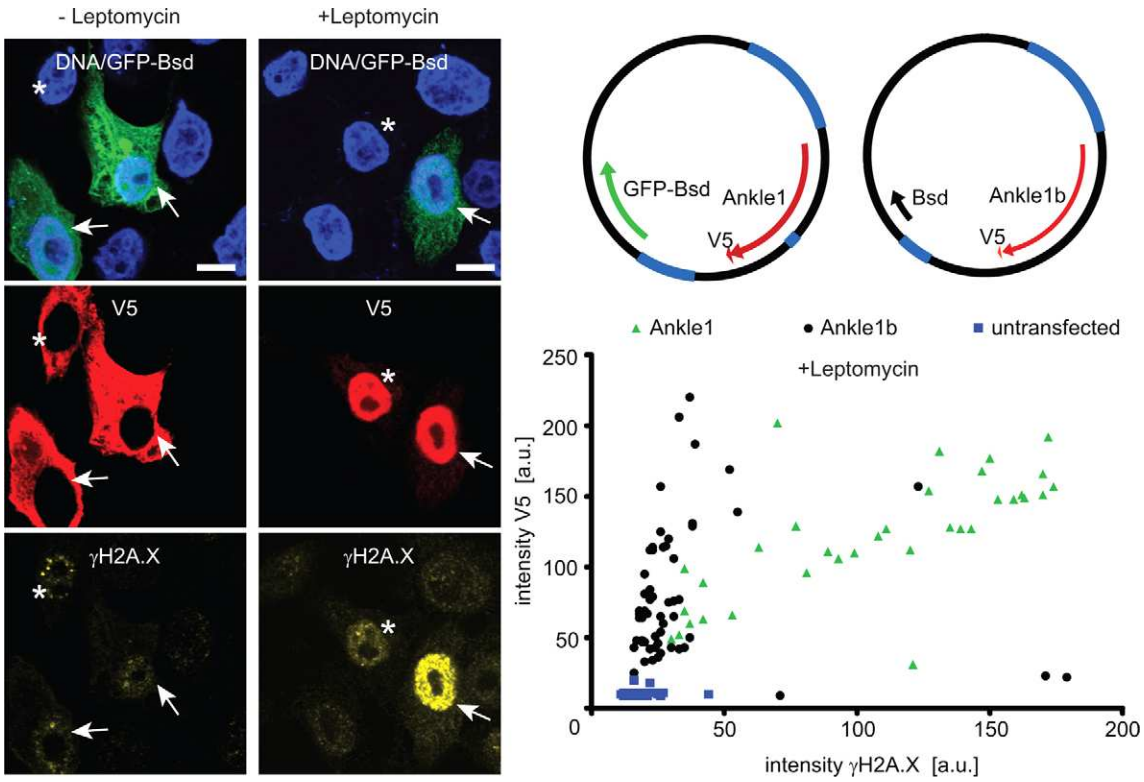

Fig. 7. LEM domain in Ankle1 is required for induction of DNA damage response. HeLa cells were transfected separately with constructs expressing Ankle1–V5 or the LEM-domain-deficient Ankle1b–V5 and seeded as a mixed culture onto coverslips after 24 hours. The plasmid expressing Ankle1–V5 also expresses a CMV-driven GFP–Blasticidin marker gene (GFP-Bsd). The plasmid encoding Ankle1b–V5 contains a Blasticidin gene without GFP fusion (Bsd) (see plasmid maps on the right). After 48 hours, cells were either treated with Leptomycin or ethanol for 3 hours, fixed, stained for V5 (red) and γH2A.X (yellow) and imaged on a confocal fluorescence microscope. DNA was stained with DAPI (blue). GFP–Blasticidin is shown in green. Cells marked with asterisks lack GFP signal and are thus expressing Ankle1b; cells expressing Ankle1 (expressing GFP) are marked with arrows. Scale bars: 10 μm. Bottom right: relative fluorescence intensities of V5 and γH2A.X signals within the nucleus of untransfected, GFP-positive and GFP-negative transfected and Leptomycin-treated cells (n>30 each) were measured and plotted using Graphpad Prism software. Representative results out of three independent experiments are shown.

including the bacterial nucleotide excision repair protein UvrC and the DNA structure-specific repair enzyme SLX1 in humans (Perrin et al., 1993; Stoddard, 2005; Truglio et al., 2005; Dunin-Horkawicz et al., 2006; Edgell, 2009; Fekairi et al., 2009; Svendsen et al., 2009). The comparison of 3D models generated from the canonical GIY-YIG homing endonuclease I-TevI and from human, *C. elegans* and hydra Ankle1 revealed striking similarities (supplementary material Fig. S3), indicating a strong structural conservation between I-TevI and Ankle1 proteins despite low primary sequence identity. Thus, Ankle1 is an unusual member of the LEM protein family and is, along with SLX1, the only active GIY-YIG-type endonuclease so far described in higher eukaryotes.

Besides the catalytically active GIY-YIG domain, homing endonucleases also contain DNA-binding motifs such as zinc-finger, α-helix and helix-turn-helix domains that position the catalytic core at the DNA. It is tempting to speculate that Ankle1 is targeted to DNA through the interaction between its LEM domain and BAF. In addition, a DNA-binding α-helix might be present within the C-terminus of Ankle1 according to in silico modeling (supplementary material Fig. S3B), but so far we have no experimental evidence for additional DNA binding motifs in Ankle1. Our hypothesis that a Ankle1–BAF complex is involved in DNA cleavage is supported by the observation that the LEM-deficient splice isoform Ankle1b, did not induce DNA damage response.

What is the physiological role of Ankle1?

When forced to the nucleus by transient inhibition of nuclear export, Ankle1 caused DNA cleavage and induced DNA damage response. In all organisms, endonucleases are instrumental in diverse DNA repair pathways (Nishino and Morikawa, 2002; Marti and Fleck, 2004). Endonucleases are also essential for the processing of double-strand breaks introduced during meiotic recombination (Borde, 2007; Mimitou and Symington, 2009) and for somatic genomic rearrangements during lymphocyte development in higher eukaryotes (Lieber et al., 2004; Rooney et al., 2004; Rivera-Munoz et al., 2007). Taking into account the predominant expression of human Ankle1 in hematopoietic tissues and in lymphoma-derived cell lines, we postulate a role of Ankle1 in genomic rearrangement or associated DNA repair processes during the development of lymphocytes. In addition, Ankle1 might have a more general function in DNA repair pathways, which could explain its conservation in lower metazoan species. Consistent with this hypothesis, others have demonstrated that loss of function of the *C. elegans* ortholog of mammalian Ankle1, *lem-3*, causes a radiation hypersensitivity (Rad) phenotype (Christina Dittrich and Michael Hengartner, University of Zurich; personal

communication). Worms carrying a leucine to phenylalanine point mutation in LEM-3 at position 659 (supplementary material Fig. S3C) are hypersensitive to DNA damage caused by chemicals and irradiation. This leucine residue is invariably conserved in all identified Ankle1 proteins at the respective positions. Interestingly, this site resides within a predicted C-terminal α-helical structure (supplementary material Fig. S3B,C) and thus could be important for Ankle1-DNA coordination. Interestingly, human Ankle1 mutated at the respective residue (L590F) did not trigger the DNA damage response pathway upon accumulation in the nucleus (supplementary material Fig. S4).

Altogether, the results indicate that Ankle1 is an evolutionary conserved GIY-YIG-type endonuclease that is predicted to have functions in DNA damage repair pathways in lower metazoan organisms (Christina Dittrich and Michael Hengartner, personal communication), whereas it seems to have acquired more specialized functions during evolution in mammals, particularly in human lymphocyte development.

Materials and Methods

Cell culture and reagents

HeLa, HEK, SW-480, T-98-G and U2-OS cells were routinely cultivated in DMEM; ARH-77, CCRF, DAUDI, K-562, VL-6, RAJI, RAMOS and REH cells were cultivated in RPMI, both supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin/streptomycin and 2 mM L-glutamine at 37℃ in a humidified atmosphere containing 8.5% CO_2 and 5% CO_2 respectively. Transient transfections were performed using Nanofectin according to the manufacturer's instructions (PAA, Pasching, Austria). Crm-dependent nuclear export was inhibited with 10 ng/ml Leptomycin B (Enzo Life Sciences, Lausen, Switzerland) in complete growth medium for 1–3 hours.

PCR analyses

Poly(A^+) RNA purified from cell lines using the mRNA isolation kit (Roche, Mannheim, Germany) or purchased human tissue total RNA samples from Agilent Technologies (Santa Clara, CA) and Biochain (Hayward, CA) were reverse transcribed using the First-strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Aliquots of the resulting products were used as templates for PCR amplification of Ankle1, actin and GAPDH using the Go-Taq PCR Master Mix (Promega, Germany) and the following primer pairs: Ankle1 forward(1) 5′-TGCCTGTGGGAGCACCAGACATC-3′, Ankle1 forward(2) 5′-GCCCTGCG-GACGGGCTGTATTC-3′, Ankle1 reverse 5′-GCTCGCCTTCAGCCAGGAA-GAC-3′, actin forward 5′-ATCTGGCACCACACCTTCTAC-3′, actin reverse 5′-CAGCCAGGTCCAGACGCAGG-3′, GAPDH forward 5′-CATCACCATCT-TCCAGGAGCGA-3′ and GAPDH reverse 5′-CCTGCTTCACCACCTTCT-TGAT-3′.

Antibodies

Mouse antibodies against V5 were purchased from Invitrogen, rabbit anti-actin from Sigma-Aldrich, rabbit anti-53BP1 and mouse anti-BAF both from Novus Biologicals (Littleton, CO), and rabbit antibody against phosphorylated Chk2 (Thr68) from Cell Signaling Technology (Boston, MA). The MANEM5 anti-emerin antibody was a kind gift from Glenn Morris, NE Wales Institute, Wrexham, UK (Manilal et al., 1996). Polyclonal rabbit anti-murine Ankle1 antibodies were raised against an N-terminal fragment encompassing amino acids 95–260, following the Austrian and European regulations on animal experimentation. Antibodies against Ankle1 were affinity purified from serum number 136 using a purified recombinant human Ankle1 fragment corresponding to mouse Ankle1 amino acids 95–260. His-tagged proteins were detected using the India HisProbe-HRP (Pierce Biotechnology, Rockford, IL).

Plasmids and cloning strategy

Human Ankle1 and the splice variant Ankle1b were amplified from bone marrow cDNA using the Platinum Pfx PCR Kit (Invitrogen). Oligonucleotides used were: Ankle1-CACC forward 5′-CACCGCTAGCATGTGCTCGGAGGCCCGCCTGG-3′ and Ankle1-wostop reverse 5′-GTATCTAGAGCCCCGGGCCTGGATGTC-3′ or Ankle1-stop reverse 5′-TCAGCCCCGGGCCTGGATG-3′. PCR products were cloned into pENTR/D-TOPO or pET102-TOPO using topoisomerase-based cloning (Invitrogen). GFP–Ankle1 fusion constructs were created by PCR amplification using primers Ankle1-*Sal*I forward 5′-AGCGTCGACAT-GTGCTCGGAGGCCCGCCTGG-3′ and Ankle1-stop reverse. The PCR product was cut with *Sal*I and ligated into *Sal*I and *Sma*I sites of the peGFP-C1 vector (Clontech, Palo Alto, CA). The deletion construct human Ankle1ΔCT (amino acids 1–420) was constructed by cloning the PCR-generated fragment into pET102-TOPO and peGFP-C1 using the same cloning strategies as described above, using the respective forward primers and the primer human Ankle1-1062-stop reverse 5′-CATTCTAGACCGGCTACAAGGGCCGACAG-3′. V5-tagged Ankle1 was generated by shuttling Ankle1 via the LR-recombination reaction (Invitrogen) from pENTR plasmids into a Gateway-compatible pTRACER plasmid (pTB) (Brachner et al., 2005) or pDEST-51 (Invitrogen). Quick-change site directed mutagenesis was performed according to the manufacturer's protocol (Stratagene, Santa Clara, CA) by amplification of pEntry-hAnkle1 with primers containing the desired mutations. Introduced point mutations were verified by sequencing, and Ankle1 mutants were shuttled into pTB or pDEST-51. The HA-Strep-Ankle1 plasmid was generated by cloning the PCR-amplified human *Ankle1* cDNA into the pENTR11 vector (Invitrogen) followed by Gateway-mediated shuttling into the pTO_HA_StrepIII_GW_FRT vector (obtained from Matthias Gstaiger, ETH, Switzerland). PCR-generated human *BAF* cDNA was cloned via the topoisomerase reaction into pET102-D-Topo for bacterial expression with a C-terminal His tag or with a downstream stop codon into pEntry-D-Topo. The pEntry-hBAF-stop was then used for Gateway cloning into the bacterial expression vector pDEST42.

Preparation of cell lysates, gel electrophoresis and immunoblotting

Cells were harvested, washed in cold PBS and resuspended in cold high-salt RIPA buffer [25 mM Tris-HCl pH 7.4, 500 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Complete protease inhibitor mix (Roche, Mannheim, Germany), 1 mM PMSF and 1 mM DTT]. Following incubation on ice for 10 minutes, cell lysates were sonicated for 5 seconds and the insoluble material pelleted. Supernatants were mixed with Laemmli sample buffer, boiled for 3 minutes and resolved via polyacrylamide gel electrophoresis. Proteins were blotted onto nitrocellulose membranes and probed with antibodies. After incubation with horse-radish-peroxidase-conjugated secondary antibodies, the Supersignal West Pico chemiluminescent substrate and CL-XPosure films (ThermoFisher Scientific, Rockford, IL) were used to detect signals.

Pull-down assay

Bacterial expression vectors containing human Ankle1a (pET102-hAnkle1a-stop or pET102-hAnkle1a-6×His-V5), human Ankle1b (pET102-hAnkle1b-6×His-V5), human Ankle1ΔCT (pET102-hAnkle1ΔCT-stop), human BAF (pET102-hBAF-6×His or pDEST42-hBAF-stop), human LAP2α (pDEST42-hLAP2α-6×His-V5) or EmerinΔTM (pET11c-EmerinΔTM, kindly provided by Kathy Wilson, Johns Hopkins University, Baltimore, MD) were transformed into BL21-star (Invitrogen). After induction of recombinant protein expression with 1 mM IPTG for 3 hours at 37℃, bacteria were lysed in RIPA buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Complete protease inhibitor mix, 1 mM PMSF and 1 mM DTT) and sonicated for 1 minute on ice. Insoluble material was pelleted and 20 µl of soluble fractions containing human Ankle1-stop, human Ankle1ΔCT-stop or EmerinΔTM were mixed with 10 µg of human BAF-6×His and incubated for 1 hour at 37℃ in 100 µl pull-down buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM imidazole, 0.5% NP-40, 1 mM EDTA and protease inhibitor mix). BAF complexes were separated with magnetic Ni^{2+}beads (Promega), washed three times with pull-down buffer, eluted by incubation with HEPES elution buffer (100 mM HEPES pH 7.4, 500 mM imidazole and 1 mM DTT) for 10 minutes and analyzed by western blotting. Pull down of untagged human BAF-stop was carried out as follows: 20 µl of lysate containing BAF-stop were mixed with 20 µl of lysate of BL21 bacteria expressing human Ankle1a-6×His-V5, human Ankle1b-6×His-V5 or human LAP2α-6×His-V5) in 100 µl of pull-down buffer and incubated for 1 hour at 37℃. Complexes were precipitated with magnetic Ni particles, washed three times with pull-down buffer and analyzed by western blotting.

Immunofluorescence

Cells were grown on poly-L-lysine-coated glass coverslips or seeded into Ibidi-treat microscopy slides for live-cell imaging (see Fig. 4A) (Ibidi, Munich, Germany). Cells on coverslips were fixed in PBS containing 4% paraformaldehyde for 10 minutes and permeabilized in PBS containing 0.5% Triton X-100 for 5 minutes. Cells were blocked in PBS containing 0.5% gelatin for 15 minutes, incubated with primary antibodies for 45 minutes, washed three times with PBS and re-probed with the appropriate secondary antibodies conjugated to either TexasRed or Cy-5 (Jackson ImmunoResearch) for 45 minutes. After three washes with PBS, DNA was counterstained with 100 ng/ml DAPI (Sigma, Munich, Germany) for 5 minutes and samples mounted in Mowiol (Fluka, Buchs, Switzerland). Images were taken using a confocal laser scanning microscope (LSM-Meta, Zeiss, Jena, Germany) using a Plan-Apochromat 63× oil immersion objective (NA 1.40). Live-cell imaging was performed on the same microscope with transfected cells seeded. Digital images were analyzed, adjusted for brightness and contrast, and mounted using the LSM Image-Browser (Zeiss, Jena, Germany) and Adobe Illustrator (Adobe Systems, San Jose, CA).

Computer-assisted analysis

Sequence alignments and database searches were performed by NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/), GraphAlign (http://darwin.nmsu.edu/cgi-bin/graph_align.cgi) (Spalding and Lammers, 2004) and ClustalW2 (http://www.ebi.ac.uk/clustalw2/) (Thompson et al., 1994). Genomic analysis was done using the ENSEMBL Genome Browser (http://www.ensembl.org/) (Sanger Institute). Additional human Ankle1 orthologs were predicted using the GENSCAN software (http://genes.mit.edu/genscan.html) (Burge and Karlin, 1997). Protein motifs and pattern searches were performed using SMART (http://smart.embl-heidelberg.de/) (Schultz et al., 1998; Letunic et al., 2004) and PSORT-II (http://psort.nibb.ac.jp/form2.html). Transmembrane domains were calculated using the TMHMM 2.0 prediction software (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) and the DAS-TMfilter algorithm (Cserzo et al., 2004). Phylogenetic tree predictions were visualized using the Phylodendron and PhyloDraw software (Choi et al., 2000).

3D modeling was performed employing the CPHmodels 3.0 Server (Nielsen et al., 2002) and visualized using the Discovery Visualizer Studio 2.5 (Accelrys Software, San Diego, CA).

COMET assay

Alkaline single cell gel electrophoresis (COMET assays) were performed as described previously (Ehrlich et al., 2008). In brief, GFP-positive HeLa cells were sorted by fluorescence-activated cell sorting (FACS), pelleted and embedded in a thin layer of low-melting agarose (Invitrogen) on microscopy slides. In order to prevent DNA damage caused by UV light, lysis and all subsequent steps were conducted under red light. After solidification of the agarose, slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10.0) at 4℃ for at least 1 hour. DNA was unwound by incubation of slides in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH≥12.5) for 20 minutes followed by electrophoresis for 20 minutes at 25 V and 300 mA. Then, the slides were neutralized by two 10-minute incubations in neutralization buffer (0.4 M Trizma base, pH 7.5) at 4℃. Slides were air-dried and stained with ethidium bromide. Formation of 'comets' was measured in randomly chosen nuclei (n>150) for each condition in three independent samples using the Comet Assay IV software (Perceptive Instruments, Haverhill, UK), and quantified and visualized in Graphpad Prism.

Endonuclease assays with HA-Strep Ankle1

HEK293 cells transiently transfected with a vector expressing HA-Strep-Ankle1 were grown on 15-cm dishes to 80% confluency. At 24 hours after transfection, the cells were pelleted by centrifugation, washed in PBS and snap-frozen. The pellets were lysed (20 mM HEPES-KOH pH 8.0, 200 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol and protease inhibitors) and incubated on ice for 20 minutes. The lysate was centrifuged and incubated with Streptactin beads (IBA biotechnology, Göttingen, Germany) for 2 hours at 4℃ on a rotating wheel (300 r.p.m.). Subsequently, the beads were washed three times with 1 ml lysis buffer and twice with 1 ml wash buffer (20 mM HEPES-KOH pH 7.5, 2 mM $MnCl_2$, 0.05 mg/ml BSA and 50 mM KCl); each wash was for 5 minutes at 4℃. HA-Strep Ankle1 was finally eluted in 100 µl of PBS containing 2.5 mM desthiobiotin (IBA Biotechnology) for 30 minutes at 4℃ on a shaker (600 r.p.m.). Aliquots were immediately snap-frozen.

For the nonspecific endonuclease assays, 80 ng of supercoiled pFastBac1 plasmid was incubated with wild-type Ankle1 or its variants in endonuclease buffer (20 mM HEPES-KOH pH 7.4, 2 mM $MnCl_2$, 45 mM KCl, 50 µg/ml BSA). After incubation at 37℃ for 1 hour, the reaction was terminated by the addition of 0.1% SDS, 14 mM EDTA and 0.1 mg/ml proteinase K and incubation at 55℃ for 15 minutes. Then, 10% glycerol was added and the samples separated on a 0.8% ethidium bromide agarose gel for 45 minutes at 80 V.

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